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EVALUATION OF A MODIFIED ENCAPSULATION–DEHYDRATION PROCEDURE INCORPORATING SUCROSE PRETREATMENTS FOR THE CRYOPRESERVATION OF *RIBES* GERMPLASM

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Summary

A modified encapsulation–dehydration cryopreservation protocol based on the replacement of cold acclimation with high-sucrose pretreatment was assessed for the long-term storage of *Ribes* germplasm. Four steps in the procedure were examined for eight genotypes: (1) pregrowth of shoot tips in sucrose-supplemented solid growth medium for 1 wk; (2) pretreatment of alginate-encapsulated shoot tips in sucrose-supplemented liquid culture medium for 21 h; (3) evaporative desiccation of encapsulated–dehydrated shoot tips; and (4) exposure to liquid nitrogen (LN). Differential responses were observed for black currant and gooseberry genotypes. Recovery of growing shoots was high (72–100%) at all four steps for the five black currants tested. Evaporative desiccation slightly decreased viability for some black currants and in some cases LN exposure reduced regrowth. In contrast, three gooseberry species had poor recovery from the initial sucrose culture step (32–67%), indicating sensitivity to osmotic stress, which predisposed these genotypes to poor survival after LN exposure (12–26%). The effectiveness of the modified protocol for conserving a wider range of *Ribes* genotypes was further ascertained by screening 22 genotypes derived from nine *Ribes* species. The procedure was successful for 18 of the 22 genotypes in the gene bank in Scotland. Screening genotype responses at the time of storage demonstrated regrowth $\geq$60% for 15 genotypes, and only four genotypes had regrowth of 0–28%. Additional genotypes were also added to the USDA cryopreserved *Ribes* collection.

Key words: black currant; conservation; gene bank; gooseberry; liquid nitrogen.

Introduction

Biotechnology plays an increasingly important role in international plant conservation programs and in preservation of the world's genetic resources (Bajaj, 1995; Callow et al., 1997; Razdan and Cocking, 1997; Benson, 1999). Traditionally, plant genetic resource management involves conserving germplasm as seed at low temperature, or for vegetatively propagated crops, as plants in field gene banks. These approaches are complemented by *in vitro* conservation methods, used in combination with traditional practices, thereby offering additional security for field gene bank conservation (Ashmore, 1997). The optimum *ex situ* genetic resource conservation program consists of active collections that make germplasm readily available for distribution or characterization, and base collections held for the sole purpose of long-term preservation.

Conservation of tissues at ultra-low temperatures (usually in liquid nitrogen (LN) at −196°C) is, theoretically, infinite. The continuing expense for germplasm maintenance is limited to the costs of securing a regular and safe supply of LN. Uniformly applicable techniques are important to the international genetic resources community and allow for the more widespread use of low-temperature storage for vegetatively propagated plants (Reed, 2001; Reed et al., 2001). Clonal germplasm storage in liquid nitrogen is now in place for dormant buds of several tree species, *in vitro* shoot tips of fruit, tuber, and grass crops, and embryonic axes from recalcitrant seed of a wide range of tropical fruits and nuts (Bajaj, 1995; Razdan and Cocking, 1997; Benson, 1999; Towill, 2002).

Several factors play a role in the successful and routine implementation of cryopreservation protocols in large-scale gene banks. When validating cryopreservation methodologies in new laboratories, source-plant status, personnel, cryogenic facilities and regimes, and culture conditions are the most likely causes of variation (Reed et al., 2001, 2004).

Cryopreservation protocols include both cryogenic (cryoprotectant and low temperature treatments) and non-cryogenic (pre- and post-storage culture) components. The success of a protocol depends on the tolerance and sensitivity of plant germplasm to the stresses accumulatively incurred at each successive stage of the cryopreservation procedure. In choosing cryoprotective treatments or protocols it is important to take into account the origin and physiological status of the germplasm (e.g., temperate or tropical,

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dormant or active), tolerance to abiotic stresses (e.g., cold and desiccation) as well as operational, technical and practical factors. To expedite the implementation of cryopreservation protocols in large-scale international gene banks it is essential to develop technically simple and robust protocols that may be applied across a wide genetic base. This ensures that cryogenic storage is cost, labor and time effective with predictable and reproducible results. To this end, vitrification-based protocols like encapsulation–dehydration are widely used to conserve a diverse range of both tropical and temperate plant germplasm (Sakai, 2004). Vitrification (the production of an amorphous glassy state) circumvents the injurious problems associated with ice formation (Benson, 2004). The encapsulation–dehydration protocol requires cryogenic storage in the total absence of ice (Benson et al., 1996). However, as vitrification involves an increase in cellular viscosity it is important that plants are able to withstand potentially lethal osmotic and evaporative dehydration stresses. Temperate species that naturally accumulate sugars and protective proteins during seasonal cold acclimation are better able to withstand stresses incurred during cryoprotection as compared to desiccation-sensitive germplasm from tropical species. Thus, it is not surprising that cold-acclimation treatments and/or treatments that simulate the biochemical base of cold acclimation have been applied with great success to enhance the cryopreservation survival of temperate and subtropical plant germplasm (Reed, 1988; Reed and Yu, 1995; Chang and Reed, 1999; Chang et al., 2000). Improving the tolerance of tropical and warm-temperate (e.g. Mediterranean species) plant germplasm to vitrification has largely been implemented through the use of high-sucrose pretreatments (Benson, 2004; Reed et al., 2004; Sakai, 2004). The possibility therefore exists of using either cold acclimation or sucrose pretreatments to achieve improved tolerance to vitrification-based cryopreservation for germplasm derived from temperate species. Sucrose pretreatments were initially explored for *Ribes* by Dumet et al. (2000a, b). Two test cultivars of *R. nigrum* responded equally well (survival 90–100%) when cryopreserved following pretreatments of cold acclimation or sucrose. The modified sucrose pretreatment protocol is based on a method initially developed by Reed and Yu (1995), who first cryopreserved shoot tips from three species of cold-acclimatized *in vitro*-grown *R. aureum* Pursh, *R. diacanthum* Pall., and *R. rubrum* L. cv. Cherry using three standard techniques. Controlled cooling and vitrification were successful for two of three genotypes while standard encapsulation–dehydration worked well for all three species. Similar results were found for *R. nigrum* L. cultivars by Benson et al. (1996). Luo and Reed (1997) developed additional improvements to the vitrification method with *R. aureum* and *R. ciliatum* Humb. and Bonpl. that led to improved recovery.

Substituting sucrose for cold-acclimation treatments may be advantageous for genotypes that are marginally or extremely chill sensitive. From a practical point sucrose pretreatment does not require the use of a cold-acclimation chamber, which may be a limiting factor in small laboratories. Thus, the objectives of the present study were to: determine the critical steps of the protocol with respect to its successful implementation for the cryostorage of diverse plant collections; test the applicability of the approach for the two major commercial groups of *Ribes*, black currants and gooseberries; ascertain the effectiveness of cryopreserving *Ribes* germplasm using a modified encapsulation–dehydration (E-D) protocol that substitutes sucrose pretreatment for cold acclimation; and explore the potential for its implementation in the creation of a small-scale gene bank for a diverse selection of *Ribes* genotypes.

Materials and Methods

Plant materials. Eight accessions from the USDA-ARS National Clonal Germplasm Repository (NCGR, Corvallis, OR, USA) *in vitro* collection were tested in replicated trials: black currant (*Ribes nigrum* L.) cultivars Malling Jet, Ojebyn, Topsy, Tenah and Willoughby; and gooseberry species *R. diacanthum*, *R. oxyacanthoides* subsp. *setosum* (Lindl.) Q. P. Sinnot, and *R. divaricatum* Douglas. Twenty *Ribes* genotypes from NCGR and two from the Scottish Crop Research Institute (SCRI, Scotland, UK) were screened with the technique (Table 1). Cultures were maintained on RIB, a modified MS medium (Murashige and Skoog, 1962), with 2% (w/v) glucose, 0.28 μM N^6-benzyladenine (BA) and 0.58 μM gibberellic acid (GA), and solidified with 0.3% (w/v) agar and 0.125% Gelrite (Reed and Yu, 1995). Shoots were cultured on 40 ml of RIB medium in Magenta boxes and maintained at 25°C with a 16-h (25 $\mu mol\, m^{-2}\, s^{-1}$) photoperiod.

Testing of modified protocol with Ribes *(black currants and gooseberries).* Shoots (1 cm) with leaves removed were cultured for 1 wk in Petri dishes containing RIB medium with 0.75 *M* sucrose, 0.8% (w/v) agar and without glucose. Shoot tips (0.8–1 mm) were excised from the conditioned shoots (Step 1; 10 shoot tips), encapsulated in alginate droplets made of low viscosity, 3% (w/v) alginic acid (Sigma Chemical, St. Louis, MO), prepared with 0.75 *M* sucrose MS basal medium free of calcium/cobalt salts, and allowed to polymerize for 20 min in a 100 m*M* $CaCl_2$ solution (Fabre and Dereuddre, 1990). Beads were transferred to 50 ml of 0.75 *M* sucrose liquid MS basal medium (40 beads per flask) and shaken at 50 rpm for 21 h (Step 2; 10 beads). After sucrose treatment the beads were dried on sterile filter paper and dehydrated under laminar flow at ambient temperature (20/25°C) (Step 3; 10 beads). The final moisture content was about 20% on a fresh weight basis. Desiccated beads were transferred to 1.2 ml plastic cryotubes (10 beads per cryotube) and plunged into LN (Step 4; 20 beads). Cryotubes were warmed for 10 min at ambient temperature (20–25°C), rehydrated for 10 min in liquid MS, and plated on medium for regrowth with 0.25% less agar and 0.1% less Gelrite than the standard RIB multiplication medium.

Viability assessment. Recovery of shoot tips was assessed at four stages of the cryopreservation process for eight genotypes: (1) after sucrose pretreatment and shoot-tip excision; (2) after Step 1 and osmotic desiccation in 0.75 *M* sucrose solution; (3) after Steps 1 and 2 and evaporative desiccation; and (4) after Steps 1–3 and exposure to liquid nitrogen. After each step, shoot tips were plated in individual cells of 24-cell plates on RIB medium. Regrowth was expressed as the percentage of shoots with new shoot growth 4 wk after thawing.

Pilot gene banking. Twenty-two genotypes were evaluated once (not replicated) for Steps 2–4 as part of the storage process. LN-exposed (1 h) meristems of each accession were warmed and plated for regrowth. Long-term storage (Scotland) of 30 shoot tips of each genotype was in six cryotubes (two canes with five beads in each tube) or 90 shoot tips for selected genotypes (USA) (10 beads each in nine cryotubes).

Results and Discussion

The modified E-D protocol was very successful for black currant germplasm (*R. nigrum* 'Malling Jet', 'Ojebyn', 'Tenah', 'Topsy', and 'Willoughby'; Figs. 1 and 2). Recovery of growing shoot tips following initial pretreatment culture on 0.75 *M* sucrose medium was 97–100% for all *R. nigrum* cultivars. Further osmotic dehydration in liquid sucrose medium for 21 h also resulted in high regrowth of the shoot tips (93–100%). A minor decrease in viability (83–100%) occurred with evaporative desiccation and in some cases a further reduction (72–100%) with LN exposure (Fig. 2*A*). In contrast, recovery of gooseberry genotypes was less promising (Fig. 2*B*), indicating susceptibility to osmotic stress in this

TABLE 1

REGROWTH AT 4 WK OF *RIBES* GERMPLASM STORAGE (SCOTLAND) CONTROL SHOOT TIPS FOLLOWING THREE CRITICAL STEPS IN THE CRYOPRESERVATION PROCESS: OSMOTIC DESICCATION IN SUCROSE FOR 21 h; EVAPORATIVE DESICCATION UNDER LAMINAR FLOW FOR 4 h; AND EXPOSURE TO LIQUID NITROGEN FOR 24 h

		Regrowth (%)[z]		
Ribes taxon	Cultivar/accession number[y]	Osmotic desiccation	Evaporative desiccation	Liquid nitrogen exposure
R. cereum Douglas (currant)	237.001	0	20	10
R. diacanthum Pallas (gooseberry)	31.001	100	90	10
Ribes species (gooseberry)	355.001	NA	100	80
R. nigrum L. (black currant)	Baldwin/746.001	NA	80	60
	Ben More	NA	NA	70
	Ben Tron	NA	100	80
	Consort/307.001	100	90	90
	Crusader/121.001	NA	70	60
	Kerry/377.001	100	100	90
	Malling Jet/376.001	100	90	60
	Ojebyn/998.001	100	100	90
	Sopiernik/426.001	100	100	50
	Tenah/410.001	40	80	50
	Topsy/5.001	80	100	70
	Willoughby/385.001	100	50	60
R. odoratum Wendl.f. (currant)	Crandal/216.001	NA	60	60
R. rubrum L. (currant)	Gloire des Sablons/314.001	100	30	40
	Portal Ruby/269.001	100	80	60
	White Dutch/387.001	100	90	80
R. sanguineum Pursh (currant)	King Edward VII/761.001	100	30	30
R. uva-crispa L. (gooseberry)	Golda/205.001	100	60	0
R. viscosissimum Pursh (currant)	281.001	100	70	80
Mean regrowth at each step for all taxa (± SD)		94.7 ± 16	75.7 ± 25.4	58.2 ± 26.3

NA, data not available.

[z] Shoot tips producing new growth at 4 wk. Based on five shoot tips of each genotype for osmotic dehydration and 10 each for evaporative desiccation and liquid nitrogen exposure.

[y] Local identifying number from the National Clonal Germplasm Repository, Corvallis, OR.

genotype group. Plantlets of all three gooseberry species were stunted on 0.75 *M* sucrose medium and the best regrowth of shoot tips following this initial step was 67% for *R. oxyacanthoides*. Recovery of *R. oxyacanthoides* shoots declined from 60% following desiccation to 22% after exposure to LN. *R. diacanthum* and *R. divaricatum* recovery was further reduced at all steps of the procedure (<40%). Osmotic shock of the shoot tip following sucrose pretreatment was evident in the stressed appearance of the plantlets (either hyperhydricity or bleaching) at the time of dissection. It is likely that cryopreservation of gooseberry genotypes is best achieved using the original cold-acclimation method. A previous study found that *R. diacanthum* cryopreserved following cold acclimation and the standard E-D protocol produced >60% regrowth after LN exposure (Reed and Yu, 1995). Thus, this comparative assessment of two *Ribes* genotype groupings clearly demonstrates the differential responses to the stresses imparted by different cryoprotective strategies (i.e., cold and desiccation tolerance).

The second part of this study involved a wider screening of representative individuals from a broader genetic group of *Ribes* accessions. While responses were expected to be more variable due to the smaller number of shoot tips (10) involved in the gene banking screen, recovery after LN exposure was acceptable (>50%) for 17 of the 22 genotypes. With few shoot tips in the gene bank (30), the required regrowth from the thawed control sample must be high (Dussert et al., 2003). In order to have one living sample recovered from a stored gene bank sample of 30 shoot tips, the minimum recovery from a control sample of 10 must be 50–60% to expect one plant from a 10 shoot-tip sample in the gene bank. In contrast, for a control sample of 20 shoot tips and a total gene bank sample of 90, only 15% regrowth is needed to assure one living propagule from a stored sample of 10. In this screen regrowth of shoot tips varied greatly among genotypes (Table 1). For most genotypes the 21-h osmotic dehydration in 0.75 *M* sucrose did not affect viability (Table 1), although responses to evaporative desiccation did vary. Most *R. nigrum* cultivars and *Ribes* species had 100% regrowth, while only 20–30% of *R. sanguineum* Pursh, *R. cereum* Douglas, and *R. rubrum* L. cv. Gloire des Sablons shoot tips tolerated this second step. At least 50% of the shoot tips of the remaining genotypes recovered from evaporative desiccation. LN exposure reduced shoot tip vitality by less than 10% for 12 of the 22 genotypes; however, a few did not recover from LN exposure using this approach. On recovery, the process did not affect culture vigor and all but the three genotypes with the least recovery (0–10%) produced actively growing shoots.

Progressive evaluation of the steps of this modified encapsulation–dehydration protocol indicates that black currants differ from other *Ribes* taxa in their tolerance to high sucrose during the

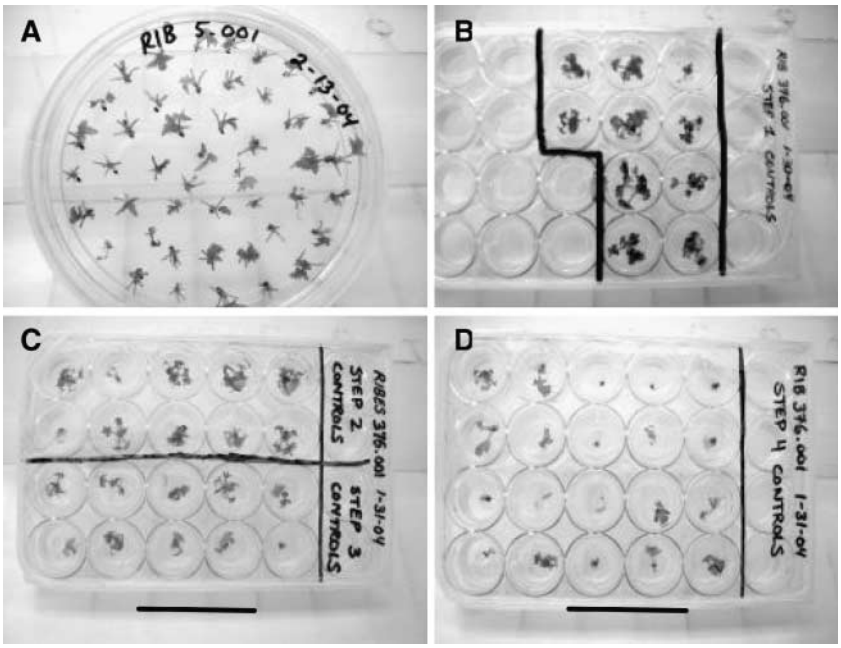

FIG. 1. Steps involved in the modified encapsulation–dehydration protocol for *Ribes*. *A*, Shoot tips from *R. nigrum* 'Topsy' *in vitro* plantlets growing on 0.75 *M* sucrose pretreatment medium (*Step 1*). *B*, Regrowth of *R. nigrum* 'Malling Jet' shoot tips 4 wk after growth on 0.75 *M* sucrose medium (*Step 1*). *C*, After alginate encapsulation and 21 h in 0.75 *M* sucrose solution (*Step 2*) and after air drying (*Step 3*). *D*, After exposure to liquid nitrogen (*Step 4*). *Bars* = 2 cm.

pretreatment phase. In several cases the other species were also sensitive to the second sucrose-desiccation step and another decrease in recovery of growing shoot tips occurred following evaporative desiccation. Declines due to evaporative desiccation and LN exposure are more likely to be due to the accumulative effects of the earlier osmotic and desiccation stresses in the protocol, influencing the ability of shoots to survive subsequent LN treatment and the secondary osmotic stresses of rewarming and recovery. It is improbable that this decline is due to differences in glass stability as influenced by genotype, since shoot tips are standardized to the same size (as confirmed by thermal analyses and water status studies) (Benson et al., 1996; Dumet et al., 2000a, 2000b).

This study demonstrated that sucrose pretreatment was effective for black currant but not gooseberry genotypes. Fluctuations in bead moisture content due to changes in environmental conditions may also influence the success of E-D techniques. Changes in ambient relative humidity could result in higher than optimum bead moisture contents so the use of silica gel or saturated salt solutions rather than air drying could possibly improve recovery of some poor-performing genotypes. Silica gel is used very effectively to desiccate tropical germplasm (Muthusamy et al., 2005). Sakai (2004) developed an encapsulation–vitrification protocol that includes initial exposure to glycerol and this protocol is effective for tropical and temperate germplasm sensitive to osmotic and dehydration stresses (Benson, 2004).

Sucrose pregrowth prior to E-D offers an alternative approach to cryopreserving *Ribes nigrum* germplasm. Screening the technique for a wide range of *Ribes* genotypes (Table 1) revealed a mean recovery across taxa of 58.2 ± 26.3 and ranging from 0 to 90%. This finding highlights the importance of studies that assess critical points in cryopreservation methods transfer between laboratories (Reed, 2001; Reed et al., 2004). It is also useful to keep in mind new probabilistic and statistical tools for screening and implementing basic cryopreservation protocols in germplasm repositories (Dussert et al., 2003; Muthusamy et al., 2005).

Large-scale cryobanking operations are required to conserve large numbers of accessions; however personnel and resources may limit the capacity to replicate tests on all accessions. Moreover, limited germplasm availability (e.g. tropical rain forest tree seeds and rare or endangered species) make it difficult to perform intensive validation tests (Benson, 2004; Muthusamy et al., 2005). Under these circumstances the sacrifice of precious and rare germplasm for extensive validation is not a favored option. Therefore, the curator can be faced with the dilemma of optimizing a method in detail using intensive replication for only one or a few genotypes and then applying it across a broader range of closely related genotypes with less intensive investigation. This may risk loss of germplasm due to lack of method robustness. Future studies on the wider implementation of cryopreservation protocols in large-scale gene banks of highly diverse germplasm may benefit from considering a three-pronged approach: (1) The use of binomial distributions to calculate the probability of recovering a fixed number of plants from a cryobank based on the regrowth of test samples (Dussert et al., 2003); (2) the determination of critical point factors in the transfer of cryopreservation technologies in international gene banks as related to effective skills training and stringent compliance with procedures (Reed et al., 2004); and (3) the use of the Taguchi experimental design to rapidly screen a wider range of cryopreservation protocol parameters with minimal sacrifice of germplasm (Muthusamy et al., 2005).

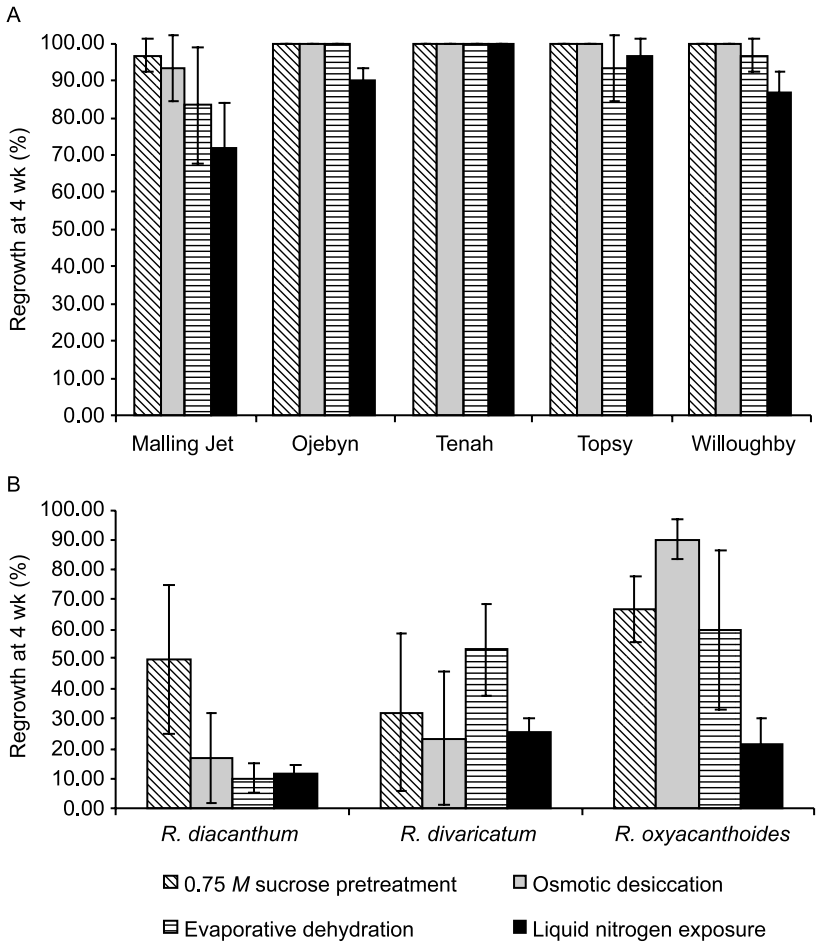

FIG. 2. Mean (± SD) recovery of *Ribes* shoot tips following each of the steps in the modified encapsulation–dehydration cryopreservation protocol. *A*, Black currant (*R. nigrum*) cultivars; *B*, gooseberry species.

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